ISO-AGGLUTINATION AND HETERO-AGGLUTINATION OF SPERMATOZOA.

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I. INTRODUCTION.

The agglutination of spermatozoa reported by Buller (1900) was first adequately described by Lillie (1913). The latter distinguished two types of agglutination differing from each other in cause and in characteristics: *iso-agglutination* produced by a substance secreted by ripe ova of the same species, and *hetero-agglutination* by substances present in egg secretions and body fluids of foreign species, Lillie (1914). The characteristics of these types will be considered in the following pages. In this

report evidence is given for the first time of the occurrence of iso-agglutination in the black chiton, *Katharina tunicata,* and of hetero-agglutination between *K. tunicata* and *S. purpuratus,* and between *K. tunicata* and *S. franciscanus,* and the reciprocal hetero-agglutination between either *S. purpuratus* or *S. franciscanus* and *K. tunicata.* Similar reciprocal hetero-agglutination occurs between either *S. purpuratus* or *S. franciscanus* and *Ishnochiton magdalenensis.*

The results embodied in this report were obtained in 1920–1921 at the Hopkins Marine Station of Leland Stanford University at Pacific Grove and at the Marine Biological Laboratory at Woods Hole. I wish to express here my appreciation of the hospitality extended to investigators at the Hopkins Marine Station and my thanks to the Director, Dr. W. K. Fisher, and to Dr. Gertrude Van Wagenen, for their assistance and encouragement. The photomicrographs accompanying this paper were taken for me by Dr. Doane, of the Department of Entomology of Stanford University. For the use of a research room at Woods Hole I am indebted to the Director, Dr. F. R. Lillie, and for suggestions and criticisms to Dr. O. C. Glaser.

II. ISO-AGGLUTINATION.

1. General.

Iso-agglutination is characterized by the rapid formation of dense spherical swarms of intensely active adherent spermatozoa and by the subsequent reversal of this process, Lillie (1921). The duration of the reaction varies with the concentration and freshness of the sperm suspension and of the egg-water, Lillie (1914, 1915), and is obtained only with motile spermatozoa, Loeb (1914). This reaction has been observed in a few marine animals and wherever it occurs indicates that we are dealing with ripe reproductive cells, species true. Its significance lies in its specificity, Lillie (1921).

Iso-agglutination has been reported by Lillie (1912, 1913) for *Arbacia punctulata* and for *Nereis;* by Glaser (1914) for *Asterias forbesii;* and by Just (1919) for *Echinarachnius.* Loeb (1914) described "cluster formation" in *Strongylocentrotus purpuratus*

and in *S. franciscanus*. This, as Lillie (1921) later determined, is identical with iso-agglutination.

2. Material and Methods.

Material.—The animals used in this work represent two phyla, Echinodermata and Mollusca. Belonging to the first are *Strongglyocentrotus purpuratus, S. franciscanus, Arbacia punctulata, Asterias ochracea,* and *Lepasterias æqualis;* and to the second *Katharina tunicata, Ishnochiton magdalenensis, Mopalia muscosa, Cryptochiton,* and *Abalone.*

Methods.—Every precaution is taken to prevent contamination of the gametes with body fluids. With the three species of sea-urchins this consists of washing animals and dissecting instruments with tap water, cutting around the oral disc and removing all body contents except the gonads, and then washing the cavity thoroughly with sea-water. The animals are then placed on their aboral surfaces in Syracuse watch glasses and allowed to shed their gametes through the germinal pores. A second method, suggested by Dr. O. C. Glaser, consists of washing the animals in tap water, rubbing off the spines, and drying with a towel. They are then allowed to shed as in the first method. Practically dry gametes can be obtained by this method. In the case of the starfish and molluscs the same method of sterilization is employed. The rays of the starfish are removed and the gonads, stripped from each ray, are transferred to a finger bowl of filtered sea-water and thoroughly washed. In the molluscs the gonads lie just beneath a dorsal shell and can be reached in one of two ways. The first consists of removing the ventral muscle and viscera with the exception of the gonads, and the second of removing the shell. The exposed gonads can then be thoroughly washed with sea-water, removed with blunt forceps to Syracuse watch glasses or finger bowls, and ruptured. In this manner dry gametes can be procured.

One (1) per cent. suspensions of spermatozoa are made by adding to 99 drops of filtered sea-water 1 drop of dry sperm. Pipettes known to deliver the same number of drops per c.c. are used. All suspensions are used within ten minutes or discarded. Standard egg-water is prepared by allowing one volume of dry

ripe eggs to secrete into two volumes of filtered sea-water for ten minutes. The egg-water is then separated from the eggs by filtration or centrifugation. The agglutination test is performed as follows: A drop of 1 per cent. sperm suspension placed between a slide and a cover glass supported by mm. glass rods spreads out into a thin film, and in it the spermatozoa may be studied microscopically. A fine-pointed capillary glass tube attached to rubber tubing is used for blowing drops of test solutions into the thin film of sperm suspension.

3. Iso-agglutination in Sea-urchins.

The results of my own experiments substantiate the statement of F. R. Lillie (1919, 1921) that egg secretions of *Arbacia, S. purpuratus,* and *S. franciscanus* produce three effects on species-true spermatozoa: *activation,* stimulation to increased motility followed later by a state of rest; *aggregation,* a tropistic phenomenon occurring only when there is a gradient from the egg-secretion to the spermatozoa; and *agglutination.*

Iso-agglutination Reaction.

An immediate and intense activation and the aggregation of the spermatozoa into a dense ring around the injected drop of egg-water is followed by the agglutination reaction. The ring rapidly becomes beaded in appearance and ultimately breaks up into small swarms. In each of the latter the spermatozoa are in such rapid motion that the entire swarm whirls about. Simultaneously similar smaller swarms form from the few spermatozoa trapped within the enclosed drop. For a brief period the swarms appear to rush together to form larger masses. The movements of the spermatozoa gradually slacken, and after a short interval, depending on the size of the swarms, a reversal occurs. They break up and in a short time the spermatozoa are dispersed, less active than originally.

In a special series of experiments I used spermatozoa of *Arbacia* from 25 per cent. and 50 per cent. suspensions which had stood at room temperature (21° C.) for from twelve to twenty-four hours, and were then aërated to remove the carbon dioxide, which in itself might affect the agglutination process. In 1 per cent. sus-

pensions of such spermatozoa I noticed an intermediate stage in the process of agglutination. This intermediate stage occurs during reversal. It is characterized by a radial orientation of the active spermatozoa such that the swarm momentarily takes on the appearance of a three-dimensional pinwheel. At the center of the wheel is a "nucleus" of sperm heads with tails radiating outward, while the periphery of the wheel is composed of a dense zone of sperm heads with their tails radiating inward. In optical section it is as though a set of spokes originating at the hub of a wheel were dovetailed between another set radiating inward from the rim. These pinwheels gradually break up and eventually the spermatozoa are completely dispersed as in typical reversible agglutination. This pinwheel formation is not comparable to a secondary aggregation which often follows iso-agglutination. In such aggregation the masses formed are irregular in shape; and the spermatozoa composing them are not intensely active, not oriented, and are readily dispersed by shaking.

4. Iso-agglutination in *Katharina tunicata*.

The spermatozoa of *K. tunicata* are inactive or but slightly active in sea-water. They can be roused to intense activity by foreign blood or foreign tissue extracts and exhibit a spiral method of locomotion similar to that described for other types of spermatozoa. In contact with surfaces they move anti-clockwise. This is probably due to their structure. (See Fig. 1.) As judged by their activation by various substances the spermatozoa of this species were ripe as early as April 9. Egg-water tested April 15, May 2, and May 16 failed to produce even an activation of spermatozoa. Inseminated eggs did not develop, indicating that the eggs were not ripe. On May 27 secretions from ripe eggs of females caught on the same day caused intense activation, aggregation, and a peculiar type of agglutination. The latter is comparable to the intermediate stage in the iso-agglutination reaction obtained with stale spermatozoa of *Arbacia*.

Following activation and aggregation of the spermatozoa into a dense ring, the latter rapidly break up into three dimensional pinwheels instead of into the whirling swarms characteristic of the iso-agglutination in sea-urchins and in *Nereis*. Within the in-

jected drop of egg-water where the spermatozoa are less concentrated it is possible to observe the process of formation of these pinwheels. A few spermatozoa first stick together in a group without any apparent orientation and without enough motility to produce whirling of the group. The three-dimensional pinwheels form instantly, including these clumps within the wheel. A fusion of pinwheels ensues for a short period. A complete reversal then occurs in some, whereas other pinwheels remain permanent. A protocol of one experiment will illustrate the time relations of the phenomenon.

Exp. 616.—Material: Doubly filtered standard egg-water of *K. tunicata;* fresh 1 per cent. sperm suspension of *K. tunicata.*
5–27/21.

3.15. Sperm suspension in sea-water—spermatozoa slightly active.
3.15. Inject a drop of egg-water—
Immediate intense activation.
Immediate ring formation.
3.16. Formation of small clumps of spermatozoa.
3.165. Formation of three-dimensional pinwheels.
3.17. Fusion of three-dimensional pinwheels.
3.19. Complete reversal of some pinwheels.
3.25. Decrease in activity; many permanent pinwheels remain.
3.40. No change.
5.00. No change. Spermatozoa still more active than originally.

On May 28 I obtained with animals brought in on the preceding day decided activation and ring formation, but no agglutination. In previous tests on spermatozoa of this species I had discovered that in order to obtain satisfactory results animals must be used on the day on which they are obtained. During June and July of 1921 and 1922 additional experiments were conducted for me by Dr. Van Wagenen. She reports results similar to those recorded in Exp. 616. However, complete reversal of agglutination was obtained with both standard egg-water and with the latter diluted ten times. Dilutions of $\frac{1}{100}$ and $\frac{1}{500}$ produced activation but no agglutination, thus indicating a rapid loss of agglutinating power with dilution.

5. Iso-agglutination in Other Echinoderms and Molluscs.

Iso-agglutination tests were made upon the spermatozoa of certain other Echinoderms and Molluscs: *Asterias ochracea,*

Lepasterias æqualis, Asterias forbesii; Ishnochiton magdalenensis, Mopalia muscosa, Cryptochiton, Abalone, and *Cumingia.* Both sperm and ova were ripe.

Activation occurred in every case, but no agglutination comparable to that in sea-urchins, *Nereis* or *K. tunicata.* In *Asterias forbesii, Asterias ochracea,* and *Cumingia* irregular clumps form consisting of a few spermatozoa. These, however, are irregularly dispersed and hence do not correspond to those which appear preceding typical iso-agglutination.

TABLE I.

THE EFFECTS OF EGG-WATER ON SPERMATOZOA OF THE SAME SPECIES.

	Spermatozoa.				
	Arbacia punctulata *Strongylocentrotus purpuratus* *Strongylocentrotus franciscanus* *Nereis limbata*	*Arbacia punctulata* (stale spermatozoa)	*Katharina tunicata*	*Asterias forbesii* *Asterias ochracea* *Cumingia*	*Lepasterias aequalis* *Ishnochiton magdalenensis* *Mopalia muscosa* *Cryptochiton* *Abalone*
Motility in sea-water.....	+	+	−	−	−
Effects of egg-water:					
Activation.....	+	+	+	+	+
Aggregation.....	+	+	+	−	−
Clumping of 3–10.....	+	+	+	+	−
Dispersal uniform.....	+	+	+	−	
Dispersal irregular....	−	−	−	+	
Agglutination-swarms ...	+	+	−	−	−
Agglutination-pinwheels	−	+	+	−	−
Reversal of agglutination:					
Partial.....	−	−	−		
Complete.....	+	+	+		
Decrease in motility.....	+	+	+	+	+
Strand formation.....	−	−	−	−	−
Cytolysis.....	−	−	−	−	−

6. DISCUSSION.

The variation in character of the iso-agglutination reaction may be due in part to the degree of motility of the spermatozoa involved. Swarming is obtained with spermatozoa highly motile in sea-water; the pinwheel type with spermatozoa inactive in sea-water (*K. tunicata*), or with spermatozoa rendered less active in sea-water by staling (*Arbacia*). The fact that spermatozoa of

both *Arbacia* and of *K. tunicata* move anti-clockwise when in contact with other objects may account for the shape of the pinwheels.

Of the ten species tested in which the spermatozoa are inactive in sea-water, iso-agglutination occurred in but one species, *Katharina tunicata.*

III. HETERO-AGGLUTINATION.

1. General.

Two distinct types of hetero-agglutination were obtained in this investigation, one with sea-urchins and one with *K. tunicata.* Hence their characteristics will be considered separately and compared with other accounts of the phenomenon.

Hetero-agglutination has been reported by Lillie (1913) between *Arbacia* egg-water or blood and *Nereis* spermatozoa; by Glaser (1914) between *Arbacia* egg-water and *Asterias* spermatozoa, and the reciprocal relationship between *Asterias* egg-water and *Arbacia* spermatozoa; by Just (1919) between *Arbacia* egg-water and *Echinarachnius* spermatozoa; and by Loeb (1914) between *S. purpuratus* egg-water and *S. franciscanus* spermatozoa. Reciprocal hetero-agglutination has been reported but once, as indicated above.

Evidence is given here of hetero-agglutination of spermatozoa of *K. tunicata* by solutions of cytolyzed spermatozoa of *S. purpuratus;* also by the blood of either *S. purpuratus* or *S. franciscanus.* The reciprocal relationship is also reported: hetero-agglutination of spermatozoa of *S. purpuratus* and of *S. franciscanus* by the blood of *K. tunicata.*

2. Material and Methods.

The material and methods include those employed in iso-agglutination experiments. In addition, blood was collected and filtered and solutions of cytolyzed spermatozoa of *S. purpuratus* and *S. franciscanus* were prepared in the following manner:

A 5 per cent. suspension of spermatozoa in glass distilled water was allowed to stand at room temperature (15° C.) for one hour, shaken at frequent intervals, and filtered (through Whatman filter paper, No. 2 and No. 50) three times.[1] In order to make

[1] Solutions made from spermatozoa which were allowed to cytolyze in dis-

the solution equal in specific gravity and hydrogen-ion concentration to sea-water, concentrated sea-water and $N/100$ NaOH were added. Controls were arranged by adding to glass distilled water concentrated sea-water and $N/100$ HCl. In making the corrections I used a standard hydrometer, and a set of standards prepared by Hynson, Westcott, and Dunning for determining the hydrogen-ion concentration of sea-water.

3. Hetero-agglutination of Spermatozoa of *Katharina tunicata.*

A. Effect of Solutions of Cytolyzed Spermatozoa of S. purpuratus.

The spermatozoa of *K. tunicata,* inactive in sea-water, are intensely activated and agglutinated by solutions of cytolyzed spermatozoa of *S. purpuratus.* The reaction resembles iso-agglutination in this species in the formation of three-dimensional pinwheels and differs from it only in the irreversibility of the hetero-agglutination (Plate I.). In forty-five experiments, in which two different test solutions were employed over a period of 34 days, similar results were obtained. A single experiment will illustrate the characteristics of the reaction.

Exp. 511.—Material: *K. tunicata* spermatozoa; solution of cytolyzed spermatozoa of *S. purpuratus.*

3–21/21—2.23 P.M.

Time.	Activation.	Agglutination.	Pinwheel Formation.	Reversal.
2.23.....	Intense	0	0	–
2.24.....	Intense	0	0	–
2.245.....	Intense	Small clumps	Few and small	–
2.25.....	Intense	0	Fusion of small clusters	0
2.45.....	Intense	0	Large and numerous	0
3.05.....	Very active	0	Large and numerous	0
3.45.....	Slightly active	0	Large and numerous	0
5 00.....	Slightly active	0	Large and numerous	0

As indicated above, the formation of pinwheels occurs after a latent period of 1½ minutes in this experiment. In other experiments the latent period was often shorter, but in no case less than 20 seconds.

tilled water from 3–5 hours caused activation but no agglutination. This may be due to an unstable property of the hetero-agglutinating substance in distilled water.

Effect of Dilution on Hetero-agglutinating Power of Solutions.—Lillie (1915) found in the case of hetero-agglutinins in *Arbacia* egg-water a disproportional loss of agglutinating power with dilution, and certain preliminary experiments with one half and one fourth dilutions of solutions of cytolyzed spermatozoa of *S. purpuratus* indicated a similar loss. The results obtained with a series of greater dilutions are indicated in the following table:

TABLE II.

THE EFFECT OF DILUTION ON STRENGTH OF HETERO-AGGLUTINATION OF SOLUTIONS OF CYTOLYZED SPERMATOZOA OF *S. purpuratus*.

	Activation.			Pinwheels.		
	Dilution.	Time.	Degree.	Time.	Number.	Size.
1.....	0	Immediate	Intense	After 20″	Many	Large
2.....	1/10	Immediate	Intense	After 4′	Many	Small
3.....	1/20	Immediate	Slight	After 2.5′	Few	Small
4.....	1/30	Immediate	Slight	After 3′	Few	Small
5.....	1/40	Immediate	Slight	After 5′	Few	Small
6.....	1/50	Immediate	Slight	After 3′	Few	Small
7.....	1/60	Immediate	Slight	After 5′	Rare	Minute

In the above summary the decrease in agglutinating power appeared to be associated with a decrease in activating power of the diluted solution of cytolyzed spermatozoa. One might predict that an activating substance added to cytolyzed sperm solutions would prevent the loss of agglutinating power. This actually proved to be true, for upon the addition of an activating body a dilution of 1/60 produced as intense and immediate activation and pinwheel formation as the undiluted solution.[1]

B. Effect of the Blood of S. purpuratus and S. franciscanus.

Filtered blood of both male and female *S. purpuratus* and *S. franciscanus* causes activation and hetero-agglutination of spermatozoa of *K. tunicata* exactly like that produced by solutions of cytolyzed spermatozoa of *S. purpuratus*. The blood must, however, be taken from the animals on the day on which they are taken from their habitat. Otherwise it will produce activation, but no agglutination. This is comparable to the deterioration of

[1] The substance in question will be discussed in a subsequent paper.

spermatozoa in animals kept in the laboratory. Blood taken from fresh animals, however, retains its hetero-agglutinating power for at least three days.

With blood, as with solutions of cytolyzed spermatozoa, there is a disproportionate loss of agglutinating power with dilution.

C. Effect of the Egg-Water of S. purpuratus and of S. franciscanus.

The egg-water of ripe ova of *S. purpuratus* and of *S. franciscanus* failed repeatedly to produce either activation or hetero-agglutination of the spermatozoa of *K. tunicata.* Considering that the latter can be intensely activated and agglutinated by blood and by solutions of cytolyzed spermatozoa of these two species of sea-urchins, and in view of the striking resemblance of iso-agglutination and hetero-agglutination in *K. tunicata,* it is especially significant that the egg-water of both species of *Strongylocentrotus* fails to produce hetero-agglutination of *K. tunicata* spermatozoa. This constitutes further evidence of the specificity of the iso-agglutinating substance present in egg-waters.

D. Changes in Spermatozoa Produced by Hetero-agglutinating Substances.

In solutions which produce hetero-agglutination the heads of the spermatozoa of *K. tunicata* become swollen at the base, as indicated in Fig. 1.

A comparison of the dimensions of spermatozoa in sea-water and in hetero-agglutinating solutions will illustrate this:

Spermatozoa.	Sea-water.	Hetero-agglutinating Solution.
Head length	10 μ	10 μ
Head width at base	2 μ	2.25 to 3.35 μ

E. Discussion.

Hetero-agglutinating substances have previously been demonstrated in blood and in egg-water. Just and Lillie have suggested that in the case of *Arbacia* the hetero-active substance is a constituent of the blood, and that egg-water used was contaminated

with blood. The hetero-active substance in the egg-water of *S. purpuratus* for *S. franciscanus* is, however, not a normal constituent of the blood of *S. purpuratus,* Lillie, 1921. It is improbable that contamination with blood can account for the strong

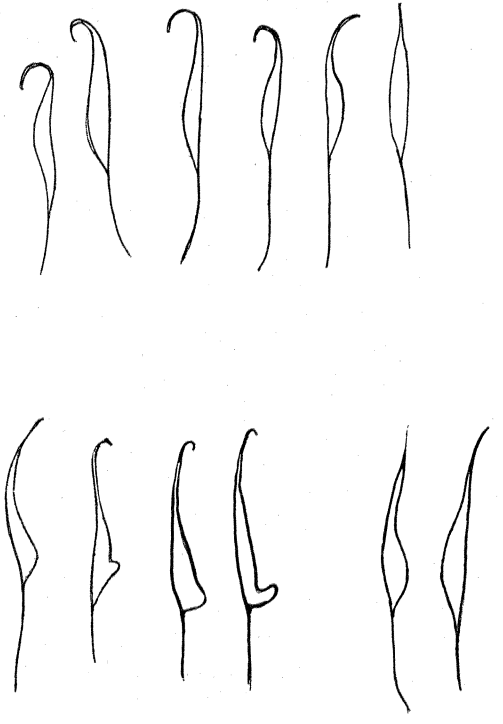

FIG. 1. Spermatozoa of *Katharina tunicata.*

a. In sea-water—normal. Mag. x 5,000 (approx.).

b. In solution of cytolyzed spermatozoa of *S. purpuratus.* The heads are swollen at the base. Mag. x 5,000.

hetero-agglutinating power of the solutions of cytolyzed sperm made from 5 per cent. suspensions of spermatozoa. Such a dilution of whole blood would have slight hetero-agglutinating properties.

It is possible that the products of cytolysis of eggs or of tissue cells of these two species of sea-urchins would also cause hetero-agglutination of spermatozoa of *K. tunicata.*

4. Hetero-agglutination of Spermatozoa of *S. purpuratus* and of *S. franciscanus* by the Blood of *Katharina tunicata.*

Since spermatozoa of *K. tunicata* were agglutinated by the blood and by solutions of cytolyzed spermatozoa of *S. purpuratus* and by the blood of *S. franciscanus,* it seemed possible that the relationship might be reciprocal. This proved to be the case, but the type of hetero-agglutination obtained resembles that described by Lillie as "mass coagulation." A single protocol will illustrate the nature of the reaction.

Exp. 585.—Blood of *K. tunicata;* spermatozoa of *S. purpuratus.* 5/4/21—2.15 P.M.

Time.	Activation.	Ring Aggregate.	Formation of		Strands.	Reversal.
			Swarms	Pinwheel.		
2.16.....	Intense	0	0	0	Few	–
2.165....	Intense	0	0	0	Many and united	0
2.25.....	Slight	0	0	0	Many and united	0

As indicated, there is no ring formation due to aggregation. The spermatozoa rapidly form strands which adhere to one another, and lose their motility. This hetero-agglutination is completely irreversible and decidedly toxic. It is of interest that the blood of *K. tunicata* which causes hetero-agglutination of spermatozoa of *S. purpuratus* and of *S. franciscanus* also causes membrane formation in the eggs of these two species. If allowed to act too long, it will induce cytolysis. A short treatment followed by a brief exposure to hypertonic sea-water will, however, lead to parthenogenetic development of the ova of *S. franciscanus,* Sampson (unpublished).

5. Hetero-agglutination in Other Echinoderms and Molluscs.

Tests were made with the spermatozoa of other Echinoderms and Molluscs to demonstrate hetero-agglutination. The results of these tests indicate two distinct types of hetero-agglutination: (*A*) the toxic "mass coagulation," described by Lillie for *Nereis* spermatozoa; (*B*) the pinwheel type, described in this report for

K. tunicata; and (*C*) a questionable third type "clumping," described by Glaser for *Arbacia.* (The "clumps" are irregularly distributed and resemble aggregation rather than hetero-agglutination.) A summary of the results is given in Tables III. and IV. These tables also include results first reported by Glaser, Just, Lillie, and Loeb, as indicated by the initials in brackets.

TABLE III.

HETERO-AGGLUTINATION OF SPERMATOZOA OF CERTAIN MARINE ANIMALS (WOODS HOLE, MASS.).

Test Solutions.	Spermatozoa.					
	Arbacia punctulata.	*Echinarachnius parma.*	*Nereis limbata.*	*Asterias forbesii.*	*Cumingia tellinoides.*	*Chiton apiculata.*
Arbacia punctulata						
Egg-water		+ *A* (J)	+ *A* (Li)	+ *C* (G)	+ *C*	–
Blood		+ *A* (J)	+ *A* (Li)			–
Sperm suspension			+ *A* (Li)			
Echinarachnius parma						
Egg-water	– (J)					
Blood	– (J)					
Nereis limbata						
Egg-water	– (Li)					
Blood	– (Li)					
Sperm suspension	– (Li)					
Asterias forbesii						
Egg-water	+ *C* (G)					
Cumingia tellinoides						
Egg-water	+ *C*					
Chiton apiculata						
Egg-water	+ *A*					
Blood						

The letters *A* and *C* refer respectively to the strand formation (Lillie, 1913) and clumping (Glaser, 1914) described in reports on hetero-agglutination.

IV. SUMMARY.

1. Iso-agglutination occurs in the black chiton, *Katharina tunicata.* This is the first report of unmistakable iso-agglutination of spermatozoa of a mollusc; also of iso-agglutination of spermatozoa which are inactive in sea-water.

2. The iso-agglutinated masses of spermatozoa of *K. tunicata* differ from those of sea-urchins and of *Nereis.* In the former the agglutinating masses resemble three-dimensional pinwheels.

TABLE IV.

HETERO-AGGLUTINATION OF SPERMATOZOA OF CERTAIN MARINE ANIMALS (PACIFIC GROVE, CALIF.).

Test Solutions.	Spermatozoa.								
	Strongylocentrotus purpuratus.	*Strongylocentrotus franciscanus.*	*Asterina.*	*Asterias ochracea.*	*Katharina tunicata.*	*Ishnochiton magdalenensis.*	*Mopalia muscosa.*	*Cryptochiton stelleri.*	*Abalone.*
Strongylocentrotus purpuratus									
Egg-water		+(Lo)	−(Lo)	−(Lo)	−	−	+C	−	−
Blood	−(Li)	−(Li)		−(Lo)	+B	+A	−	−	−
Cytolyzed sperm	−	−		−	+B	−	−	−	−
Strongylocentrotus franciscanus									
Egg-water	−(Lo)		−(Lo)	−(Lo)	−	−	−	−	−
Blood	−(Lo)	−(Lo)		−(Lo)	+B	+A	−	−	−
Asterina									
Egg-water	−(Lo)	−(Lo)							
Asterias ochracea									
Egg-water	−(Lo)	−(Lo)							
Blood	−(Lo)	−(Lo)		−(Lo)					
Katharina tunicata									
Egg-water	+A	+A		−					
Blood	+A	+A		+C	−				
Ishnochiton magdalenensis									
Egg-water									
Blood	+A	+A				−			
Mopalia muscosa									
Egg-water									
Blood							−		
Cryptochiton stelleri									
Egg-water									
Blood								−	
Abalone									
Egg-water									
Blood									−

The letters used refer to types of hetero-agglutination (*A*) to strand formation; (*B*) to pinwheel formation; and (*C*) to clumping. The latter may be considered as aggregation rather than as a type of hetero-agglutination. See text.

In these the spermatozoa are so oriented that the center consists of a nucleus of sperm heads with tails radiating outward, and the periphery is composed of a dense zone of sperm heads with tails radiating inward.

In sea-urchins and in *Nereis* the iso-agglutinating masses resemble whirling swarms in which the spermatozoa either are not oriented or are oriented with their heads forming the central nucleus of the swarm. However, a pinwheel type of iso-agglutination can be obtained with stale *Arbacia* spermatozoa. The motility of the latter is decidedly subnormal. It is possible that the variation in shape of agglutinated masses may be correlated with the degree of motility of the spermatozoa which are involved.

3. Unmistakable iso-agglutination can not be detected in any of the following: *Asterias ochracea, Asterina, Asterias forbesii, Lepasterias æqualis, Cumingia, Ishnochiton magdalenensis, Mopalia muscosa, Cryptochiton,* and *Abalone.*

4. Two types of hetero-agglutination are here reported: toxic "mass coagulation," similar to that described by Lillie (1913) for *Nereis* and by Just (1919) for *Echinarachnius,* and a pinwheel type in *Katharina tunicata,* which bears a startling resemblance to iso-agglutination in the same species.

5. Hetero-agglutination occurs between spermatozoa of *K. tunicata* and solutions of cytolyzed spermatozoa or of blood of either *S. purpuratus* or of *S. franciscanus.* The reciprocal also occurs: hetero-agglutination of spermatozoa of either *S. purpuratus* or of *S. franciscanus* by the blood of *K. tunicata.*

6. Hetero-agglutination of spermatozoa of *Ishnochiton magdalenensis* may be produced by the blood of either *S. purpuratus* or of *S. franciscanus.* The reciprocal may also be produced: hetero-agglutination of spermatozoa of either *S. purpuratus* or of *S. franciscanus* by blood of *Ishnochiton magdalenensis.*

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Plate I.

Photomicrographs of Hetero-agglutination of Spermatozoa of *Katharina tunicata* in a solution of cytolyzed spermatozoa of *Strongylocentrotus purpuratus*.

The spermatozoa form three-dimensional pin-wheels.

Magnification × 60.
Magnification × 260.